Antistaphylococcal Activities of the New Fluoroquinolone JNJ-Q2[▽][§]

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The new broad-spectrum fluoroquinolone JNJ-Q2 displays *in vitro* activity against Gram-negative and Gram-positive organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA) and ciprofloxacin-resistant MRSA isolates. Tested with isogenic methicillin-susceptible *S. aureus* (MSSA) and MRSA strains bearing quinolone-resistant target mutations, JNJ-Q2 displayed MICs ≤ 0.12 µg/ml, values 16- to 32-fold lower than those determined for moxifloxacin. Overexpression of the NorA efflux pump did not impact JNJ-Q2 MICs. Inhibition of *S. aureus* DNA gyrase and DNA topoisomerase IV enzymes demonstrated that JNJ-Q2 was more potent than comparators against wild-type enzymes and enzymes carrying quinolone-resistant amino acid substitutions, and JNJ-Q2 displayed equipotent activity against both enzymes. In serial-passage studies comparing resistance selection in parallel MRSA cultures by ciprofloxacin and JNJ-Q2, ciprofloxacin readily selected for mutants displaying MIC values of 128 to 512 µg/ml, which were observed within 18 to 24 days of passage. In contrast, cultures passaged in the presence of JNJ-Q2 displayed MICs ≤ 1 µg/ml for a minimum of 27 days of serial passage. A mutant displaying a JNJ-Q2 MIC of 4 µg/ml was not observed until after 33 days of passage. Mutant characterization revealed that ciprofloxacin-passaged cultures with MICs of 256 to 512 µg/ml carried only 2 or 3 quinolone resistance-determining region (QRDR) mutations. Cultures passaged with JNJ-Q2 selection for up to 51 days displayed MICs of 1 to 64 µg/ml and carried between 4 and 9 target mutations. Established *in vitro* biofilms of wild-type or ciprofloxacin-resistant MRSA exposed to JNJ-Q2 displayed greater decreases in bacterial counts (7 days of exposure produced 4.5 to >7 $\log_{10}$ CFU decreases) than biofilms exposed to ciprofloxacin, moxifloxacin, rifampin, or vancomycin.

Although methicillin-resistant *Staphylococcus aureus* (MRSA) has been a problematic nosocomial pathogen since the 1960s, in recent years the number of cases of community-associated MRSA (CA-MRSA) infections has increased significantly (13, 21). The majority of invasive MRSA cases now occur in the community (21). U.S. hospital admissions for acute bacterial skin and skin structure infections (ABSSSI) increased approximately 29% between 2000 and 2004, with a disproportionate increase in admission of younger patients (13). Community-associated MRSA strains, exemplified by the predominant USA300 clone, express a combination of antibiotic resistance and virulence traits that have facilitated their increased potency in this setting (5, 11). Community MRSA isolates (in particular, USA300 isolates) are increasingly multidrug resistant, with resistance profiles recently broadening to include clindamycin, tetracycline, mupirocin, and fluoroquinolone agents, in addition to the β-lactams (32). Occasionally, community isolates also display reduced susceptibility to vancomycin or resistance to gentamicin or trimethoprim-sulfamethoxazole (32).

These resistance trends necessitate the development of newer antimicrobial agents to effectively treat infections caused by resistant pathogens, including MRSA; there is, in particular, a need for oral agents that can be administered in a community setting. The fluoroquinolone agents levofloxacin and moxifloxacin displayed improved potency against Gram-positive pathogens (22), although these activities were insufficient to support their use in treating infections caused by MRSA. These newer fluoroquinolones also tend to display balanced activities in the inhibition of the essential enzymes DNA gyrase and DNA topoisomerase IV (18). The rapid emergence of ciprofloxacin resistance in staphylococci (3) may be attributable to dissimilar potencies in the inhibition of the topoisomerase targets (15), combined with the selection of efflux-based resistance (27). New fluoroquinolone agents active against ciprofloxacin-resistant MRSA would likely display equipotent activities against staphylococcal DNA gyrase and DNA topoisomerase IV and not serve as a substrate for efflux pumps. Several investigational fluoroquinolones have demonstrated *in vitro* activity against MRSA and are reported to be the subject of clinical studies to evaluate their efficacy in the treatment of ABSSSI, including infections caused by MRSA (2, 4, 20, 22; W. O'Riordan et al., presented at the Nineteenth European Congress on Clinical Microbiology and Infectious Diseases, Helsinki, Finland, 16 to 19 May 2009. Resistance selection is expected to present a challenge to the development and clinical utility of new fluoroquinolone agents in the treatment of MRSA infections. JNJ-Q2 is a new fluoroquinolone that exhibits activity *in vitro* against Gram-negative and Gram-positive pathogens, including levofloxacin-resistant and multidrug-resistant *Streptococcus pneumoniae* isolates and *S. aureus*, including ciprofloxacin-resistant MRSA isolates (14, 24).

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Herein we report the activities of JNJ-Q2 and comparator fluoroquinolone agents against methicillin-susceptible *S. aureus* (MSSA) and MRSA carrying defined target mutations and upregulated efflux, as well as the inhibition of *S. aureus* wild-type and mutant DNA gyrase and topoisomerase IV enzymes. The results of studies on the comparative selection of resistance in MRSA through serial passage in the presence of JNJ-Q2 or ciprofloxacin and the activities of these agents in established models of MRSA *in vitro* biofilms are also presented.

In an accompanying article, the *in vivo* activities of JNJ-Q2 are presented, demonstrating efficacy in murine models of MSSA and MRSA septicemia, *S. pneumoniae* lower respiratory tract infection, and in acute and established models of MRSA skin infection (14a).

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MATERIALS AND METHODS

Antimicrobial agents. JNJ-Q2, or 7-[(3E)-3-(2-amino-1-fluoroethylidene)-1-piperidinyl]-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid, was synthesized at Johnson & Johnson Pharmaceutical Research and Development (J&JPRD). Moxifloxacin and levofloxacin were obtained from Bayer AG and Ortho-McNeil, respectively. Ciprofloxacin, norfloxacin, rifampin, vancomycin, tetracycline, ethidium bromide, and reserpine were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO). Nadifloxacin and clinafloxacin were purchased from LKT Laboratories (St. Paul, MN).

Bacterial isolates. *S. aureus* strains ATCC 29213 and ATCC 43300 were obtained from the American Type Culture Collection. Mutants derived from these strains were generated for this study.

In vitro **susceptibility.** MICs were determined in accordance with the appropriate Clinical and Laboratory Standards Institute (CLSI) methodology (6) by either the broth microdilution method, using cation-adjusted Mueller-Hinton medium (MH), or the MH agar (MHA) dilution method (7). Reserpine, an inhibitor of the NorA efflux pump and perhaps of other major facilitator superfamily (MFS)-class transporters in *S. aureus* (28), was used at a fixed final concentration of 20 μg/ml, when present. The quality control (QC) strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were included as controls in susceptibility tests.

Selection and characterization of mutant strains. *S. aureus* strains ATCC 29213 and ATCC 43300 were cultured on selective media as follows: mutations in *parC* were selected on MHA containing ciprofloxacin, mutations in *gyrA* were selected on nadifloxacin, and *norA* mutants were selected on MHA containing norfloxacin plus ethidium bromide. Approximately 10^{10} CFU/plate were inoculated onto 100-mm agar plates and incubated at 37°C for 24 to 72 h. Mutations in the quinolone resistance-determining region (QRDR) of *parC* (*grlA*), *parE* (*grlB*), *gyrA*, and *gyrB* and the regulatory region of *norA* were determined from PCR products. Primers used for PCR amplification are listed in Table 1. PCR products were sequenced at ACGT, Inc. (Wheeling, IL), using the PCR primers. The relative levels of *norA* expression in selected mutants were assessed through quantitative reverse transcription-PCR (RT-PCR) and were normalized to 16S rRNA expression. RT-PCR was performed using an Applied Biosystems 7300 instrument (Foster City, CA). RT-PCR primers are listed in Table 1.

DNA gyrase and topoisomerase IV enzyme inhibition. PCR with a high-fidelity polymerase (Invitrogen, Carlsbad, CA) was used to amplify subunit DNA from *S. aureus* MRSA N315 prior to cloning into expression vectors. Site-directed mutagenesis was used to introduce fluoroquinolone-resistant mutations into cloned genes (QuikChange; Agilent Technologies, Santa Clara, CA). GyrA and ParC and their mutant counterparts, GyrA S84L and ParC S80F, were constructed as N-terminal glutathione *S*-transferase (GST)-6× His fusion proteins in pET42. GyrB and ParE were constructed as N-terminal 6× His-tagged proteins in pET28. Plasmids were transformed into *Escherichia coli* One Shot TOP10 or *E. coli* XL Gold cells for cloning and *E. coli* Rosetta2 BL21(DE3) pLysS cells for expression of the *S. aureus* DNA gyrase and DNA topoisomerase

TABLE 1. Primers for PCR and RT-PCR

Name	Primer sequence	Source or reference
gyrA PCR Forward	GCGATGAGTGTTATCGTTGCT	This study
gyrA PCR Reverse	CAGGACCTTCAATATCCTCC	This study
gyrB PCR Forward	CAGCGTTAGATGTAGCAAGC	29
gyrB PCR Reverse	CCGATTCCTGTACCAAATGC	29
parC PCR Forward	GATGAGGAGGAAATCTAG	This study
parC PCR Reverse	GTTGGAAAATCAGGACCTT	This study
parE PCR Forward	GACAATTGTCTAAATCACTTGTA	This study
parE PCR Reverse	CATCAGTCATAATAATTACAC	This study
norA PCR Forward	TGTTAAGTCTTGGTCATCTGCA	This study
norA PCR Reverse	AGCAGCAACAAGTAACCCTAAA	This study
norA RT-PCR Forward	ATCGGTTTAGTAATACCAGTCTTGC	12
norA RT-PCR Reverse	GCGATATAATCATTTGAGATAACGC	12
16S rRNA RT-PCR Forward	CGGTACCTAATCAGAAAG	27
16S rRNA RT-PCR Reverse	TTTCCAGTTTCCAATGAC	27

IV subunits. All constructs were confirmed by DNA sequencing of both strands by ACGT, Inc. (Wheeling, IL).

Protein expression was induced in 2 liters of culture with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 25°C for 3 h. Cell pellets were suspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 300 mM NaCl, 5% glycerol, 1 mg/ml lysozyme, complete EDTA-free protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]), incubated on ice for 1 h, sonicated on ice, and centrifuged at $30,000 \times g$ 45 min. The enzyme subunits were purified using Talon (cobalt-based) resin and eluted with imidazole. ProTEV protease (Promega) was used to cleave the GST-His tag from GyrA and ParC. Purified proteins were dialyzed overnight in 50% glycerol–25 mM Tris-HCl (pH 7.5)–500 mM NaCl before being subjected to flash freezing and storage in aliquots at −80°C. The purity of the enzyme subunits ranged from 74% to 92%, as determined by densitometric scanning of Coomassie-stained polyacrylamide gel electrophoresis (PAGE) gels.

Subunits for DNA gyrase and DNA topoisomerase IV were combined at various molar ratios and tested for maximal activity. For wild-type DNA gyrase, maximal activity was observed at a 1:2 GyrA/GyrB molar ratio; for mutant DNA gyrase, the maximal activity was obtained with a 1.5:1 molar ratio. For both wild-type and mutant DNA topoisomerase IV, the maximal activity was obtained with a 2.5:1 molar ParC/ParE ratio. Reconstituted enzyme aliquots were stored at −80°C. Specific activities for the wild-type DNA gyrase and mutant DNA gyrase were 390/μg and 420 units/μg, respectively, and specific activities for the wild-type DNA topoisomerase IV and mutant DNA topoisomerase IV were 39/μg and 91 units/μg, respectively. A unit value of 1 was defined as representing the minimal amount of enzyme needed to supercoil and relax 200 ng of relaxed pBR322 at 1 h and 37°C for DNA gyrase and DNA topoisomerase, respectively.

The assay was modified from the method of Robertson et al. (26) except that relaxed pBR322 (Topogen) (0.2 μg/reaction) was used. Reaction mixtures were loaded onto 1.2% agarose gels in 1× TAE (Tris-acetate-EDTA–ethidium bromide [0.5 μg/ml] in gel and buffer) and run at 70 V for 3.5 h. The gels were destained in water for 15 to 30 min before analysis. The gels were analyzed using Bio-Rad Quantity One software. Background was subtracted by the rolling disk method. The CC_{50} value, defined as the concentration inhibiting 50% of the maximum cleavable complex formation, was determined by nonlinear regression, with the upper limit representing the maximum percent cleavage obtained in the assay. Assays with maximum cleavage < 10% or with R^2 values < 0.75 were discarded.

Serial passage studies. Selection of mutants with reduced susceptibility to test agents was performed using five independent broth cultures of *S. aureus* ATCC 43300 (labeled series A through E) serially passaged in either ciprofloxacin or JNJ-Q2 continuously throughout the course of the study, without purification of single colonies in between passages. Bacteria were initially inoculated into broth containing no agent or 0.25×, 0.5×, 1×, 2×, or 4× the MIC of either ciprofloxacin (MIC = 0.5 μg/ml) or JNJ-Q2 (MIC = 0.004 μg/ml). At each passage step, the inoculum was taken from the tube containing the highest concentration of agent and supporting growth that matched the no-agent control. Approximately 5×10^7 cells were transferred at each passage step. In a few cases, isolates were frozen as glycerol stocks and subsequently restarted in fresh broth to

TABLE 2. *In vitro* activity of JNJ-Q2 and comparators against *S. aureus* MSSA and MRSA wild-type strains and
defined isogenic quinolone-resistant mutants

Strain	Genotype[a]	MIC[b] (μg/ml)				
		JNJ-Q2	Moxifloxacin	Nadifloxacin	Ciprofloxacin	Norfloxacin
S. aureus MSSA ATCC 29213	Wild type	0.004	0.06	0.03	0.25	1
	$parC^{S80F}$	0.008	0.12	0.06	2	16
	$gyrA^{S84L}$	0.015	0.06	0.12	0.5	1
	$norA^{UP}$	0.008	0.06	0.03	4	8
	$parC^{S80F}gyrA^{S84L}$	0.12	2	2	32	128
	$norA^{up}/parC^{S80F}$	0.008	0.25	0.03	8	64
	$norA^{up}/gyrA^{S84L}$	0.015	0.25	0.12	2	8
	$norA^{up}/parC^{S80F}/gyrA^{S84L}$	0.12	4	2	128	>256
S. aureus MRSA ATCC 43300	Wild type	0.004	0.03	0.03	0.5	1
	$parC^{S80F}$	0.008	0.12	0.03	4	32
	$gyrA^{S84L}$	0.015	0.06	0.12	0.5	2
	$norA^{UP}$	0.008	0.06	0.03	4	8
	$parC^{S80F}/gyrA^{S84L}$	0.12	2	2	32	128
	$norA^{up}/parC^{S80F}$	0.015	0.25	0.03	16	64
	$norA^{up}/gyrA^{S84L}$	0.015	0.06	0.12	4	4
	$norA^{up}/parC^{S80F}/gyrA^{S84L}$	0.12	2	2	256	>256

[a] NorA was overexpressed at 16-fold and 49-fold in the mutants derived from ATCC 29213 and 43300, respectively.
[b] The presence of reserpine (20 μg/ml) reduced the MICs for ciprofloxacin and norfloxacin only for those strains overexpressing NorA.

continue serial passage. Mutants were passaged at least twice in the absence of selection prior to characterization for susceptibility or mutations.

Mutations in the *gyrA*, *gyrB*, *parC*, and *parE* target genes of selected serial-passage isolates were characterized by DNA sequence analysis of PCR products amplified from QRDR and full-length regions of target genes. Primers for PCR amplification and sequencing were from Schmitz et al. (29) or Strahilevitz et al. (31) or were designed for this study.

***In vitro* biofilm studies.** Biofilm studies were modified from the *in vitro* colony biofilm model described by Stewart and colleagues (1, 35). Briefly, four 13-mm-diameter, 0.1-μm-pore-size polycarbonate membrane filters (Whatman Nuclepore membranes; catalog no. 110405) were overlaid on a 25-mm-diameter, 0.2-μm-pore-size polycarbonate membrane filter (Whatman Nuclepore membranes; catalog no. 110606) on Mueller-Hinton agar. Each 13-mm-diameter disk was inoculated in the center with 2 μl of a cell suspension in saline solution containing approximately 10^6 CFU of the test organism. Colony biofilms were grown on the disks in the absence of antibiotic at 37°C for 4 days. Membranes were then transferred to Mueller-Hinton agar containing the indicated antibiotic and incubated for an additional 7 days, with daily transfer of the disks to fresh agar medium without selection or with JNJ-Q2 (MIC, 0.004 μg/ml), moxifloxacin (MIC, 0.06 μg/ml), ciprofloxacin (MIC, 0.5 μg/ml), rifampin (MIC, 0.015 μg/ml), or vancomycin (MIC,1 μg/ml). Antibiotic concentrations of 0.25 μg/ml or 2 μg/ml (see Fig. 2A) or multiples (1×, 4×, 16×, or 64×) of the ATCC 43300 MIC values (JNJ-Q2, moxifloxacin, and vancomycin only; see Fig. 2B). The fixed concentration of 2 μg/ml was selected to approximate clinical peak plasma levels for the maximum concentrations of the free, unbound fraction of the drug in serum (fC_{max}) for moxifloxacin, ciprofloxacin, and rifampin (9, 16, 33, 34), as estimated from total drug concentrations by the use of protein binding values of 50%, 30%, and 80%, respectively (9), and 0.25 μg/ml was selected arbitrarily as a low concentration; these two fixed concentrations were chosen to evaluate effects on biofilms at clinically relevant comparator concentrations. Testing of antibiotics for biofilm efficacy at multiples of the MIC was performed to evaluate compounds at comparable levels of potency relative to their respective MICs. Vancomycin, with an fC_{max} of 33 μg/ml (50% protein binding) (17), was evaluated at 2× its clinical peak serum level in experiments performed with biofilms at 64× the drug MIC (64 μg/ml). Following 3 or 7 days of incubation on antibiotic-containing agar, each harvested 13-mm-diameter disk was subjected to a vortex procedure for 5 min in 1 ml of sterile saline to suspend cells, and the saline solution was diluted in 10-fold increments in microtiter plates. The CFU were measured by spotting 8 μl of each dilution on charcoal agar (Becton Dickinson; catalog no. 289410); CFU were estimated from the dilution exhibiting approximately 5 to 40 colonies. The limit of detection for most experiments was 125 CFU.

For enumeration of *S. aureus* cells within the biofilm population expressing reduced antibiotic susceptibility, selected dilutions of cell suspensions from day-11 biofilms exposed to antibiotic at 2 μg/ml were plated on Mueller-Hinton

agar containing 4× the MIC of the respective antibiotic and incubated (24 h at 37°C, followed by 24 h at room temperature), and the numbers of resistant colonies were counted.

RESULTS

JNJ-Q2 and comparator fluoroquinolone activities against *S. aureus* strains carrying defined target mutations. Clinical strains of *S. aureus* have previously been shown to exhibit reduced susceptibility to fluoroquinolones, typically through Ser84Leu and Ser80Phe substitutions in DNA gyrase and DNA topoisomerase IV, respectively (19, 29). Strains carrying these mutations, present either singly or in combination in MSSA ATCC 29213 and MRSA ATCC 43300, were used to assess the impact on JNJ-Q2 and comparator fluoroquinolone activity. In addition, the effect of overexpression of the NorA efflux pump was also tested.

JNJ-Q2 displayed lower MICs against wild-type *S. aureus* MSSA ATCC 29213 and MRSA ATCC 43300 than comparator fluoroquinolones moxifloxacin, nadifloxacin, ciprofloxacin, and norfloxacin (Table 2). A single Ser80Phe ParC substitution increased JNJ-Q2 MICs by 2-fold, from 0.004 to 0.008 μg/ml, in both MSSA and MRSA backgrounds. Moxifloxacin and nadifloxacin MICs were either unchanged or elevated 2- to 4-fold with this ParC substitution, in contrast to ciprofloxacin and norfloxacin, which displayed 8- to 32-fold-higher MIC values. A single Ser84Leu GyrA substitution elevated JNJ-Q2 MICs by 4-fold, from 0.004 to 0.015 μg/ml, in both MSSA and MRSA backgrounds, a result similar to those seen with moxifloxacin and nadifloxacin, for which MICs were unchanged or likewise elevated 2- to 4-fold. MIC values for ciprofloxacin and norfloxacin activity against the single GyrA mutant were unchanged or elevated 2-fold. Strains carrying both ParC Ser80Phe and GyrA Ser84Leu substitutions displayed JNJ-Q2 MICs 32-fold higher than the MICs determined for wild-type MSSA and MRSA, as with moxifloxacin and nadifloxacin, which displayed MICs elevated 32- to 128-fold. Ciprofloxacin

TABLE 3. CC_{50} values for DNA gyrase and topoisomerase IV enzymes from wild-type and quinolone-resistant *S. aureus* strains[a]

Agent	DNA gyrase CC_{50} (μg/ml)		Mutant/wild-type DNA gyrase ratio	Topoisomerase IV CC_{50} (μg/ml)		Mutant/wild-type topoisomerase IV ratio
	Wild type	S84L mutant		Wild type	S80F mutant	
JNJ-Q2	0.010	0.15	15	0.005	0.13	26
Clinafloxacin	0.045	1.2	27	0.020	0.16	8
Nadifloxacin	0.056	23	411	0.040	6.3	157
Moxifloxacin	0.33	4.0	12	0.023	4.2	183
Levofloxacin	0.086	26	302	0.085	9.8	115
Ciprofloxacin	1.8	>1,000	>555	0.26	43	165

[a] CC_{50} values are presented as the means of the results of three independent determinations.

and norfloxacin displayed MICs against the double mutants that were elevated 64- to 128-fold above the MIC values seen with the two parental strains. Based on these activities, the tested fluoroquinolones comprise two groups, with ciprofloxacin and norfloxacin MIC values elevated more by ParC than GyrA substitutions and JNJ-Q2, moxifloxacin, and nadifloxacin MIC values elevated more by GyrA than ParC substitutions.

In MSSA and MRSA mutants overexpressing NorA, the MICs for JNJ-Q2, moxifloxacin, and nadifloxacin were either unchanged or elevated 2-fold relative to the parental strains. The MICs of JNJ-Q2, moxifloxacin, and nadifloxacin against single and double ParC and GyrA mutants were unaffected by NorA overexpression (Table 2). In contrast, overexpression of NorA raised the MICs for ciprofloxacin and norfloxacin 8- to 16-fold above the MICs seen with the parental strains and 2- to 8-fold above the MICs seen with the single and double ParC and GyrA mutants.

Activities against wild-type DNA gyrase and topoisomerase IV enzymes purified from *S. aureus*. The inhibition of *S. aureus* wild-type and mutant DNA gyrase and DNA topoisomerase IV enzymes by JNJ-Q2 and comparator fluoroquinolones was assessed through biochemical assays. JNJ-Q2 was the most potent compound against wild-type DNA gyrase and DNA topoisomerase IV, with CC_{50} values of 0.01 and 0.005 μg/ml, respectively (Table 3). The comparator fluoroquinolones displayed DNA gyrase CC_{50} values ranging between 0.05 and 1.8 μg/ml and DNA topoisomerase IV CC_{50} values ranging between 0.02 and 0.26 μg/ml. With purified enzymes carrying amino acid substitutions known to confer reduced fluoroquinolone susceptibilities, CC_{50} values for all tested fluoroquinolones increased between 8 and >500-fold (Table 3). Despite these raised values, JNJ-Q2 remained the most potent fluoroquinolone, with CC_{50} values of 0.15 and 0.13 μg/ml against mutant DNA gyrase and DNA topoisomerase IV, respectively. The CC_{50} values for JNJ-Q2 increased above those for wild-type enzymes by 15-fold for mutant DNA gyrase (Ser84Leu) and 26-fold for mutant DNA topoisomerase IV (Ser80Phe), comparable to the 27- and 8-fold increases observed for clinafloxacin. CC_{50} values for moxifloxacin increased 12-fold for mutant DNA gyrase but increased 183-fold for mutant DNA topoisomerase IV. Nadifloxacin, levofloxacin, and ciprofloxacin CC_{50} values for both mutant enzymes each increased >100-fold above the values for wild-type enzymes.

Characterization of JNJ-Q2 and comparator fluoroquinolones in serial passage studies. Five independent cultures of *S. aureus* MRSA ATCC 43300 were serially passaged in the presence of increasing concentrations of either ciprofloxacin or

JNJ-Q2, and the stepwise increases in MIC for each culture are presented in Fig. 1. Wild-type ATCC 43300 displayed a ciprofloxacin MIC of 0.5 μg/ml, and MIC increases of 32-fold (to 16 μg/ml) were observed in all five cultures by 9 to 16 days of serial passage (Table 4). Further MIC increases to 128 and 512 μg/ml were observed for all five cultures by day 18 to 24 of passage. The ciprofloxacin-resistant mutants each displayed JNJ-Q2 MICs of ≤0.25 μg/ml and moxifloxacin MICs of 2 to 4 μg/ml. Wild-type ATCC 43300 displayed an MIC of 0.004 μg/ml for JNJ-Q2; in contrast to the results of serial passage in the presence of ciprofloxacin, each of the five cultures passaged in the presence of JNJ-Q2 displayed MICs of ≤1 μg/ml for a minimum of 27 days, with two cultures not displaying MICs of ≥1 μg/ml for a minimum of 49 days (Table 5). MIC increases to 0.125 to 0.5 μg/ml (increases of 32- to 128-fold) were selected between days 12 and 25 of serial passage. No mutant displaying a JNJ-Q2 MIC of 4 μg/ml was observed until after 33 days of passage, and the highest JNJ-Q2 MICs observed, 32 and 64 μg/ml, were not selected until 49 and 51 days of passage, respectively. Mutants selected through serial passage in the presence of JNJ-Q2 also displayed elevated MICs of the comparator fluoroquinolones, with ciprofloxacin and moxifloxacin MICs increasing to a maximum of 128 μg/ml and 4 to 512 μg/ml, respectively, for each of the five independent cultures.

Susceptibility tests performed in the presence and absence of the efflux pump inhibitor reserpine indicated that JNJ-Q2 did not select for upregulated efflux through inhibition of pumps by reserpine (e.g., NorA). For terminal isolates of each JNJ-Q2 series, the inclusion of reserpine did not reduce the MIC value for JNJ-Q2, ciprofloxacin, or ethidium bromide (data not shown). Likewise, tetracycline MICs did not increase in terminal isolates, indicating that the MepA efflux pump (23) was not overexpressed in JNJ-Q2-selected mutants.

Characterization of target mutations in serial-passage isolates. Serial passage in the presence of ciprofloxacin selected for well-characterized target mutations in the QRDRs of DNA gyrase and DNA topoisomerase IV associated with resistance to existing fluoroquinolone agents (19). The observed mutations included Ser84Leu and Ser80Phe substitutions in GyrA and ParC, respectively (Table 6). A single ParC Glu84Lys substitution was observed to raise the ciprofloxacin MIC to 16 μg/ml, and terminal ciprofloxacin-passaged isolates displayed drug MICs of 256 to 512 μg/ml and carried 2 to 3 QRDR mutations, including substitutions in active site serine residues of both target enzymes. Com-
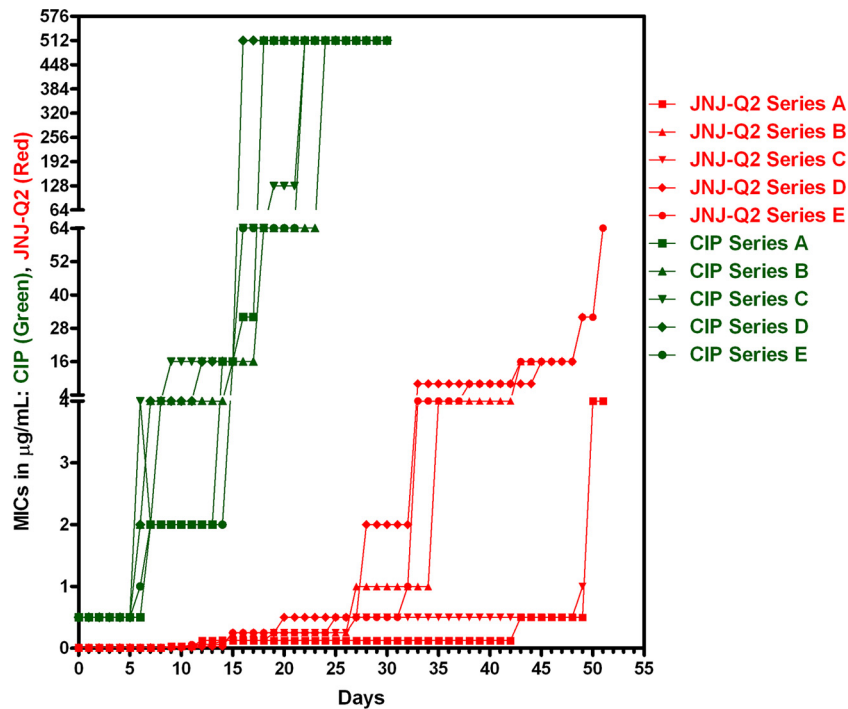
FIG. 1. Increase in MIC value for *S. aureus* MRSA ATCC 43300 serially passaged in the presence of either ciprofloxacin (CIP) or JNJ-Q2. Bacteria were initially inoculated into broth containing no agent or 0.25×, 0.5×, 1×, 2×, or 4x the MIC of either ciprofloxacin or JNJ-Q2. Approximately 5×10^7 cells were transferred at each passage step.

mon QRDR mutations were also identified in JNJ-Q2-selected mutants, including Ser84Leu, Ser85Pro, and Glu88Lys mutations in GyrA and Ser80Phe, Glu84Lys, and His103Tyr mutations in ParC. In each of the five independent series,

JNJ-Q2 selected for mutants carrying Glu422Asp and/or Asp432Val/Gly substitutions in ParE as early as 13 days of passage. Fewer substitutions were observed in GyrB; those substitutions included Asp433Glu, Ile450Val, and

TABLE 4. Ciprofloxacin-selected mutants in *S. aureus* ATCC 43300 resulting from serial passage

Strain category or CIP series	Day of passage	MIC^a (µg/ml)					
		CIP	JNJ-Q2	MXF	NAD	CLX	NOR
ATCC 43300 wild type		0.5	0.004	0.06	0.015	0.03	1
CIP series							
A	7	1	0.004	0.125	0.03	0.06	8
	14	4	0.015	0.25	0.03	0.25	16
	16	16	0.015	0.25	0.03	0.5	64
	18	512	0.25	4	2	4	>512
B	6	2	0.015	0.125	0.06	0.06	32
	7	4	0.015	0.125	0.06	0.06	32
	15	16	0.06	0.5	0.25	0.25	64
	18	64	0.25	2	2	1	64
	24	512	0.125	2	2	1	128
C	6	4	0.015	0.125	0.06	0.06	16
	9	16	0.03	0.25	0.06	0.5	128
	16	64	0.06	2	2	2	512
	19	128	0.12	2	1	1	256
	22	512	0.12	2	0.5	2	>512
D	6	2	0.015	0.125	0.06	0.06	8
	7	4	0.015	0.125	0.06	0.06	32
	15	16	0.03	1	0.25	0.25	32
	16	256	0.25	4	2	4	>512
E	7	2	0.015	0.125	0.03	0.06	32
	15	16	0.03	0.125	0.06	0.125	64
	16	64	0.25	2	2	4	64
	22	256	0.25	2	2	2	512

^{a} CIP, ciprofloxacin; MXF, moxifloxacin; NAD, nadifloxacin; CLX, clinafloxacin; NOR, norfloxacin.

TABLE 5. JNJ-Q2-selected mutants in *S. aureus* ATCC 43300 through serial passage

Strain category or JNJ-Q2 series	Day of passage	MIC[a] (µg/ml)					
		CIP	JNJ-Q2	MXF	NAD	CLX	NOR
ATCC 43300 wild type		0.5	0.004	0.06	0.015	0.03	1
JNJ-Q2 series							
A	9	1	0.015	0.25	0.25	0.125	4
	12	2	0.125	1	0.5	0.25	4
	13	128	0.125	4	1	1	256
	43	64	0.5	4	8	2	256
	51	32	2	32	16	16	125
B	9	0.5	0.03	0.25	0.25	0.125	2
	12	2	0.125	0.5	0.5	0.25	8
	28	128	1	16	16	8	256
	36	128	4	512	64	128	256
	44	128	16	512	64	128	512
C	13	1	0.125	0.25	0.25	0.25	8
	27	4	0.5	2	1	0.5	16
	49	4	1	4	2	1	16
D	12	2	0.03	0.5	0.125	0.25	32
	15	64	0.25	4	4	2	128
	20	64	0.5	8	4	4	256
	29	128	2	16	8	8	256
	34	128	8	256	128	128	256
	49	128	32	512	128	128	256
E	12	1	0.06	0.5	0.25	0.125	4
	25	64	0.5	4	0.5	2	256
	33	128	4	64	4	16	256
	43	128	16	256	16	128	256
	51	128	64	512	64	128	256

[a] CIP, ciprofloxacin; MXF, moxifloxacin; NAD, nadifloxacin; CLX, clinafloxacin; NOR, norfloxacin.

Gln476Lys, identified in isolates passaged between 44 and 51 days, although the impact on the fluoroquinolone susceptibility of these mutations is unknown. Likewise, the potential contribution of the Gln6His substitution in ParC, observed in two independent series, to fluoroquinolone resistance is also unknown. Terminal isolates selected by passage in JNJ-Q2 carried between 4 and 9 target enzyme substitutions and displayed JNJ-Q2 MICs of 1 to 64 µg/ml (Table 6).

For the terminal isolates selected by JNJ-Q2, the entire open reading frames for the four genes encoding the target enzymes DNA gyrase and topoisomerase IV were sequenced to potentially identify mutations outside of the QRDR that may contribute to reduced susceptibility. ParE His101Asn substitutions were identified in two independent JNJ-Q2-selected terminal isolates, after 44 and 49 days of serial passage (data not shown). The only additional non-QRDR mutation identified was a ParE Ser141Asn in the series C, day 49 isolate (data not shown). The potential contribution of these mutations to resistance is unknown, and no additional mutations outside of the QRDR were identified for terminal isolates for series A, D, and E. No significant differences were observed in the apparent *in vitro* fitness of mutant strains compared with the ATCC 43300 parent strain (data not shown).

Activity of JNJ-Q2 and comparator fluoroquinolones against *in vitro* MRSA biofilms. Prior to treatment with antibiotics, viable cells of *S. aureus* biofilms increased from the starting inoculum of approximately 10^6 CFU to approximately 2×10^9 CFU by day 4, with 90% of the growth occurring within the first 24 h (data not shown). This pre-

drug incubation period was followed by incubation for an additional 7 days in the presence of a selective agent at the indicated fixed concentrations (Fig. 2A) or at multiples ($1\times$, $4\times$, $16\times$ or $64\times$) of the MIC (JNJ-Q2, moxifloxacin, and vancomycin only; Fig. 2B). Data from enumeration of cells within untreated and treated wild-type *S. aureus* MRSA ATCC 43300 biofilms are presented in Fig. 2A and B. Untreated control biofilms maintained constant bacterial counts of approximately 2×10^9 CFU between days 4 and 11 of culture. After 3 or 7 days of treatment with vancomycin (MIC, 1 µg/ml) at concentrations $\leq$ 64 µg/ml, colony biofilms exhibited little or no decrease in cell counts (0.0 to 0.4 $\log_{10}$ CFU) relative to contemporary untreated control biofilms (Fig. 2B). Treatment of colony biofilms with rifampin (MIC, 0.015 µg/ml) at concentrations of 0.25 to 64 µg/ml for 3 days yielded $\log_{10}$ CFU decreases of 0.8 to 1.4. Notably, after treatment of biofilms with rifampin for 7 days, a partial recovery of cell numbers was observed such that bacterial counts in rifampin-treated biofilms at day 7 were only 0.5 $\log_{10}$ below those in untreated control biofilms. In contrast to vancomycin and rifampin results, exposure of biofilms to JNJ-Q2 (MIC, 0.004 µg/ml) at fixed concentrations of 0.25 and 2 µg/ml produced $\log_{10}$ reductions in biofilm CFU of 2.8 and 3.6, respectively, following 3 days exposure, and the biofilm counts were reduced >7 $\log_{10}$, to below the limit of detection (125 CFU), following 7 days of exposure (Fig. 2A). Ciprofloxacin (MIC, 0.5 µg/ml) produced biofilm CFU decreases of 0.2 to 1.3 $\log_{10}$ at 3 days of exposure and 0 to 2.4 $\log_{10}$ at 7 days of exposure to concentrations ranging from 0.25 to 4 µg/ml (data not shown). Bacterial counts were 1 $\log_{10}$ higher in ciprofloxacin-treated biofilms (2 µg/

TABLE 6. Mutations selected in genes encoding target topoisomerases in ciprofloxacin- or JNJ-Q2-passaged strains

Selecting agent and series	Day of passage	MIC (μg/ml)		Topoisomerase mutation(s)[a]			
		CIP	JNJ-Q2	GyrA	GyrB	ParC	ParE
CIP							
A	18	512	0.25	Ser84Leu	WT[b]	Ser80Phe	WT
B	24	512	0.125	Ser84Leu	WT	Ser80Phe	WT
C	22	512	0.125	Gly82Cys	WT	Ser80Phe	WT
D	15	16	0.03	WT	WT	Glu84Lys	WT
	16	256	0.25	Ser84Leu	WT	Ser80Phe, Glu84Lys	WT
E	26	256	0.06	Ser84Leu	WT	Ser80Phe	WT
JNJ-Q2							
A	9	1	0.015	Ser84Leu	WT	Gln6His	WT
	13	128	0.25	Ser84Leu	WT	Ser80Phe	WT
	43	64	0.5	Ser84Leu, Glu88Lys	WT	Ser80Phe	WT
	51	32	2	Ser84Leu, Glu88Lys	WT	Ser80Phe, His103Tyr	Glu422Asp, Asp432Gly
B	9	0.5	0.03	Ser84Leu	WT	WT	WT
	28	128	1	Ser84Leu, Glu88Lys	WT	Glu84Lys, His103Tyr	WT
	36	128	4	Ser84Leu, Glu88Lys	WT	Ser80Phe, Glu84Lys	WT
	44	128	16	Ser84Leu, Glu88Lys	Asp433Glu	Ser80Phe, Glu84Lys, His103Tyr	Glu422Asp
C	13	1	0.125	Ser84Leu	WT	WT	Asp432Val
	27	4	0.5	Ser84Leu, Ser85Pro	WT	WT	Asp432Val
	49	4	1	Ser84Leu, Ser85Pro	WT	WT	Glu422Asp, Asp432Val
D	12	2	0.03	WT	WT	Ser80Phe	WT
	15	64	0.25	Ser84Leu	WT	Ser80Phe	WT
	29	128	2	Ser84Leu, Glu88Lys	WT	Ser80Phe	Asp432Asn
	49	128	32	Ser84Leu, Glu88Lys	Ile450Val	Ser80Phe, Ser81Pro	Asp432Asn
E	25	64	0.5	Ser84Leu	WT	Ser80Phe	WT
	32	128	1	Ser84Leu	WT	Gln6His, Ser80Phe, Glu84Lys	WT
	33	128	4	Ser84Leu, Glu88Lys	WT	Gln6His, Ser80Phe, Glu84Lys	WT
	43	128	16	Ser84Leu, Ser85Pro, Glu88Lys	WT	Gln6His, Ser80Phe, Glu84Lys	WT
	51	128	64	Ser84Leu, Ser85Pro, Glu88Lys	Gln476Lys	Gln6His, Ser80Phe, Glu84Lys, His103Tyr	Glu422Asp

[a] The QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* were sequenced for all isolates, and the entire open reading frames of these genes were sequenced for the terminal isolate in each of the JNJ-Q2 serial-passage series.
[b] WT, wild-type sequence.

ml) at day 7 in comparison with day 3, indicating regrowth of the biofilm. This was not observed in biofilms treated with ciprofloxacin at 4 μg/ml (data not shown). Exposure of biofilms to moxifloxacin (MIC, 0.06 μg/ml) at 0.25 and 2 μg/ml reduced biofilm CFU by 0.8 and 1.7 $\log_{10}$, respectively, following 3 days of exposure, and by 0.4 and 5.8 $\log_{10}$, respectively, following 7 days of exposure (Fig. 2A). Exposure of biofilms at 1$\times$, 4$\times$, or 16$\times$ the JNJ-Q2 or moxifloxacin MICs demonstrated similar reductions in CFU numbers for either agent (Fig. 2B). At 1$\times$ or 4$\times$ the MIC, neither fluoroquinolone caused a measurable reduction in biofilm CFU numbers of ATCC 43300. At 16$\times$ the MIC, JNJ-Q2 and moxifloxacin reduced biofilm CFU numbers by $\leq$1.2 $\log_{10}$ following treatment for 3 or 7 days. JNJ-Q2 and moxifloxacin at 64$\times$ the MIC (0.25 and 2 μg/ml, respectively) reduced ATCC 43300 biofilm CFU numbers by 2.8 and 1.7 $\log_{10}$ after 3 days of treatment and by >7 $\log_{10}$ and 5.8 $\log_{10}$ after 7 days treatment (Fig. 2B).

The results of treatment of established biofilms composed of fluoroquinolone-resistant mutants derived from ATCC 43300 were also assessed. Mutants carrying a single Ser80Phe ParC substitution or a single Ser84Leu GyrA substitution or a combination of the two mutations displayed JNJ-Q2 MICs of 0.015, 0.015, or 0.5 μg/ml, respectively, and moxifloxacin MICs of

0.125, 0.125, or 2 μg/ml, respectively. Exposure of established biofilms of the single ParC mutant, the single GyrA mutant, and the double mutant to JNJ-Q2 at 2 μg/ml (133$\times$ the single-mutant drug MIC and 4$\times$ the double-mutant drug MIC) produced bacterial biofilm reductions of 7.0, 5.8, and 4.5 $\log_{10}$ CFU, respectively, following 7 days of exposure (Fig. 2B). In contrast, exposure of biofilms of the single ParC mutant, the single GyrA mutant, and the double mutant to moxifloxacin at 2 μg/ml (16$\times$ the single-mutant drug MIC and 1$\times$ the double-mutant drug MIC) for 7 days resulted in bacterial biofilm reductions 0.5, 0.3, and 0.1 $\log_{10}$ CFU, respectively (Fig. 2B).

Resistance selection in biofilms exposed to each antibacterial agent was assessed through plating samples on agar containing 4$\times$ the MIC of the specific agent. Biofilms of wild-type 43300 exposed to either rifampin or ciprofloxacin contained more than 1×10^9 colonies per biofilm that displayed resistance to the selecting agent at both days 3 and 7 of exposure. The presence of high numbers of resistant cells correlated with the apparent regrowth of biofilms treated with rifampin or ciprofloxacin. Rifampin-resistant colonies were also detected in untreated control biofilms at frequencies of 3×10^{-7} and 5×10^{-7} at days 3 and 7, respectively. No vancomycin-resistant colonies were detected in vanco-
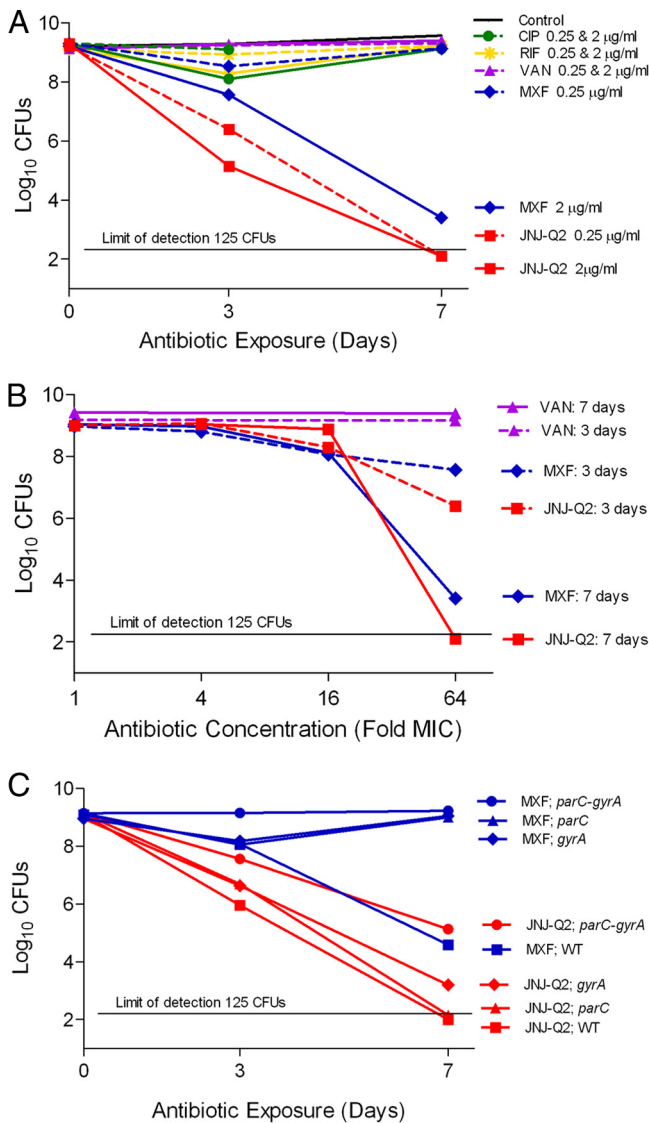
FIG. 2. (A) Activities of JNJ-Q2 and comparators against *in vitro* biofilms of *S. aureus* MRSA ATCC 43300 at 0.25 μg/ml and 2 μg/ml. (B) Activities of JNJ-Q2 and comparators against *in vitro* biofilms of *S. aureus* MRSA ATCC 43300 at 1×, 4×, 16×, and 64× the MIC. (C) Activities of JNJ-Q2 and moxifloxacin (at 2 μg/ml) against *in vitro* biofilms of fluoroquinolone-resistant mutants of *S. aureus* MRSA ATCC 43300. CIP, ciprofloxacin; RIF, rifampin; VAN, vancomycin; MXF, moxifloxacin.

mycin-treated biofilms. No resistant colonies were identified in biofilms treated with JNJ-Q2 or moxifloxacin at 2 μg/ml for either 3 or 7 days. Limited resistance to JNJ-Q2 was noted at 4× or 16× the MIC (0.015 and 0.06 μg/ml), with MICs increasing two- or 4-fold. No data are available for moxifloxacin at these multiples of the MIC.

DISCUSSION

The increasing prevalence of MRSA as a causative agent of infections in the community setting, including invasive disease, along with the increasingly multidrug resistant pro-

files of community MRSA isolates (32) presents the need for new agents, particularly orally active agents, that can be used to treat these infections. The apparent elevation in the fitness of resistant, community-associated MRSA isolates may eventually result in the displacement of traditional nosocomial isolates within health care institutions, potentially resulting in an increased severity of infections and longer hospitalizations (10). Currently, no members of the fluoroquinolone class are approved for the treatment of infections caused by MRSA or fluoroquinolone-resistant pathogens. Medicinal chemistry advances in the synthesis of new fluoroquinolones have generated agents that display *in vitro* activity against ciprofloxacin-resistant streptococci and staphylococci, including MRSA, and are reported to be currently undergoing evaluation in clinical studies for the treatment of skin infections. These new agents include the fluoroquinolone delafloxacin (4; O'Riordan et al., unpublished) and the des-fluoroquinolone nemonoxacin (22).

The new broad-spectrum fluoroquinolone JNJ-Q2 demonstrated antistaphylococcal activities, with MIC_{90} values of ≤ 0.25 μg/ml against collections of *S. aureus*, MRSA, and methicillin-resistant *S. epidermidis* (MRSE), including contemporary ciprofloxacin-resistant MRSA and MRSE isolates (14, 24). In this study, in tests performed using a set of mutants derived from MSSA ATCC 29213 and MRSA ATCC 43300 that carried common fluoroquinolone-resistant mutations in the target genes, JNJ-Q2 was the most active agent, with MIC values 16- and 32-fold lower than nadifloxacin and moxifloxacin and at least 1,024-fold lower than ciprofloxacin and norfloxacin. A mutation upregulating the NorA efflux pump alone or in combination with these target site mutations did not elevate MIC values for JNJ-Q2.

The MIC results presented here as determined with characterized MRSA mutants suggested that JNJ-Q2 retained activity against ciprofloxacin-resistant strains as a result of potent inhibition of DNA gyrase and possibly DNA topoisomerase IV as well. This suggestion was supported by the results of *in vitro* inhibition of purified *S. aureus* DNA gyrase and DNA topoisomerase IV, for which the CC_{50} values for JNJ-Q2 were comparable. The relative activities of JNJ-Q2 against enzymes carrying common active site mutations associated with fluoroquinolone resistance further supported this hypothesis in that single-target active-site substitutions in either DNA gyrase or DNA topoisomerase IV elevated the CC_{50} values for JNJ-Q2 to roughly the same extent. This was not the case for the comparator fluoroquinolone agents, several of which demonstrated roughly equal potency against wild-type enzymes but disproportionately elevated CC_{50} values for one of the target enzymes carrying a mutation conferring fluoroquinolone resistance. An exception was clinafloxacin, which, like JNJ-Q2, displays roughly equipotent activities against both target enzymes (25). The lower MIC values for JNJ-Q2, in comparison with other tested fluoroquinolone agents, against wild-type and fluoroquinolone-resistant MSSA and MRSA correlated with lower CC_{50} values for JNJ-Q2, suggesting intrinsic tight binding to the enzyme targets within bacterial cells.

Ciprofloxacin resistance in staphylococci rapidly emerged following the introduction of ciprofloxacin into the clinic (3). New fluoroquinolone agents developed to treat staphy-

lococcal infections need to demonstrate a reduced propensity for resistance selection, particularly in MRSA isolates. In a direct comparison of resistance selection in MRSA during serial passage in the presence of ciprofloxacin versus JNJ-Q2, resistance was rapidly selected by ciprofloxacin but not by JNJ-Q2, for which serial-passage isolates displayed JNJ-Q2 MIC values ≤ 1 μg/ml through at least 28 days of serial passage. High-level ciprofloxacin resistance was associated with only 2 or 3 target mutations, while elevated MICs in JNJ-Q2-selected mutants were associated with 4 to 9 target mutations. This finding is consistent with the equipotent activity of JNJ-Q2 against the target enzymes and its likely tight association with both DNA gyrase and DNA topoisomerase IV. Characterization of resistant mutants selected by ciprofloxacin and JNJ-Q2 demonstrated active site substitutions in the target enzymes that are typically associated with fluoroquinolone resistance (8, 19), and this was reflected in the elevated MICs for comparator fluoroquinolones in mutants selected by either agent. Although the first mutation identified in 4 of 5 JNJ-Q2-passaged cultures was in *gyrA*, suggesting that GyrA is the preferred target of JNJ-Q2 within bacterial cells, the first JNJ-Q2-selected mutation mapped to *parC* in one series, supporting the equipotent activity observed for the purified enzymes. This result was in contrast to those observed for ciprofloxacin, which displayed greater potency against purified DNA topoisomerase IV and selected for first-step mutations only in *parC*. One JNJ-Q2-selected mutation, a Gln6His substitution in ParC, has not been previously characterized as contributing to reduced fluoroquinolone susceptibility in *S. aureus*, and although the impact of this mutation on JNJ-Q2 MIC values is unknown, the mutation was selected in two independent serial-passage cultures and is potentially indicative of unique contacts for JNJ-Q2 with DNA topoisomerase IV.

Bacteria within biofilms display reduced susceptibility to antibacterial agents of many different chemical classes, including vancomycin (30). JNJ-Q2 and moxifloxacin reduced ATCC 43300 biofilm CFU numbers to similar levels when tested at multiples of 1×, 4×, and 16× the MIC, but JNJ-Q2 was more effective than moxifloxacin or ciprofloxacin at fixed concentrations (0.25 and 2 μg/ml) that approximate clinical plasma levels for the latter two clinically approved fluoroquinolones. The greater potency of JNJ-Q2, relative to the potency of comparators that included vancomycin, rifampin, ciprofloxacin, and moxifloxacin, in reducing or eliminating wild-type and resistant MRSA biofilms, was consistent with its lower *in vitro* MIC and indicates a potential for this agent in the treatment of device-related infections. The *in vitro* antistaphylococcal activities of JNJ-Q2, including greater potency against purified target enzymes and wild-type and resistant MRSA strains in established biofilms and a reduced potential for resistance selection, likely reflect potent and balanced inhibition of DNA gyrase and topoisomerase IV combined with the negligible impact of efflux on JNJ-Q2. This profile is consistent with the properties required for the development of an antistaphylococcal fluoroquinolone and support continued investigation of this agent.

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